

Molecular cloning of a major *Alternaria alternata* allergen, rAlt a 2

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Background: Sensitivity to the fungus *Alternaria alternata* is a common cause of asthma. Epidemiologic studies from a variety of locations worldwide indicate that *A alternata* sensitivity is closely linked with the development of asthma. Furthermore, *A alternata* sensitivity has been associated with severe and potentially fatal attacks of asthma.

Objective: The diagnosis of *A alternata* sensitivity is hampered by the lack of standardized and well-characterized allergenic extracts. Molecular cloning of allergens offers the possibility of providing large quantities of purified, well-characterized allergens not only for diagnostic purposes but also for studying the pathogenesis of *A alternata* sensitivity. We used molecular cloning to identify, purify, and produce a major *A alternata* allergen in quantity.

Methods: We prepared messenger (m)RNA from *A alternata* to produce a complementary (c)DNA library. The library was screened for *A alternata* allergens by using sera from *A alternata*-sensitive individuals. A recombinant allergen was isolated, the cDNA sequence was determined, and the protein was expressed in *Pichia pastoris*.

Results: A unique *A alternata* allergen, rAlt a 2, was identified. A 2.2-kb cDNA sequence was obtained that has homology with a common transposable region and mouse RNA-dependent eukaryote initiation factor-2 α -kinase but no homology to any known allergen. No N-glycosylation sites were found in the cDNA sequence. The recombinant allergen was recognized by IgE antibodies in the sera of 16 of 26 (61%) individuals allergic to *A alternata*, which defines Alt a 2 as a major allergen.

Conclusions: We have molecularly cloned a unique major *A alternata* allergen, rAlt a 2. Identification and expression of the recombinant allergen should enhance the development of standardized *A alternata* allergenic extracts and provide stable reagents for investigating the pathogenesis of *A alternata* sensitivity. (*J Allergy Clin Immunol* 1999;104:665-71.)

Key words: *Alternaria alternata*, recombinant allergens, rAlt a 2

Alternaria alternata is a major aeroallergen in many regions of the world.^{1,2} Although the precise prevalence of *A alternata* sensitivity is not known, an epidemiologic study of over 17,000 US citizens demonstrated positive skin test responses to *A alternata* in 3.6%,³ and up to

Abbreviation used

TBS: TRIS-buffered saline

70% of patients with mold allergy have skin test reactivity to *A alternata*.⁴ Furthermore, *A alternata* sensitivity has been shown to be a risk factor for asthma.⁵⁻⁷ In addition, *A alternata* sensitivity can also lead to severe and fatal asthma.⁸ *A alternata* sensitivity is therefore an important clinical problem.

Studies of *A alternata* sensitivity have been complicated by the complexity and variability of fungal extracts and by the lack of standardized material for diagnosis and treatment. Yunginger et al⁹ found that commercially available *A alternata* extracts varied as much as 300-fold in potency. Vijay et al¹⁰ found a 40-fold variation of potency in batch-to-batch preparations from one commercial source. In addition, individual strains of *A alternata* produce variable quantities of allergens depending on the growth conditions of the organism.¹¹ A number of allergens have been purified biochemically from *A alternata*.¹²⁻¹⁹ The most well known of these allergens is Alt a 1. The N-terminal amino acid sequence of this protein has been obtained.¹⁹ However, no standardized *A alternata* extracts are available, in part because of the lack of purified, well-characterized allergens in quantity.

Because it has been difficult to obtain purified allergens in quantity by biochemical methods, molecular biology techniques, including molecular cloning of allergens, have been used to obtain allergens for investigation.²⁰ Subunits of Alt a 1 have been cloned.^{21,22} Furthermore, 3 other *A alternata* allergens (Alt a 6, Alt a 7, and Alt a 10) have been molecularly cloned, and their complementary (c)DNA sequences have been determined.² These recombinant allergens are minor allergens based on IgE immunoblotting studies that indicated the prevalence of sensitivity to these allergens is low (8%, 7%, and 2%, respectively).² Enolase, a highly conserved fungal protein, has been cloned and identified as a major allergen in *A alternata* and *Cladosporium herbarum*.²³

Cloning of major *A alternata* allergens (ie, allergens that are recognized by IgE antibodies in $\geq 50\%$ of sensitive patient sera) is needed to further understand mechanisms of *A alternata* sensitivity and improve the quality of diagnosis and therapeutic reagents.

In this study we report the construction of a cDNA library and gene cloning, high-level expression, and

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characterization of a unique, major allergen of *A alternata*. This allergen has been designated rAlt a 2 in accordance with the International Union of Immunological Societies Allergen Nomenclature Subcommittee's recommendation.^{24*}

METHODS

Establishment of a cDNA library

A alternata ATCC strain 46582 was cultured at room temperature in stationary Roux bottles containing modified Czapek's medium.²⁵ After 5 days of growth, the mats were obtained by filtration. Five grams of the mats were frozen at -80°C and pulverized. Total cellular RNA was obtained by extracting the powder with guanidine isothiocyanate-mercaptoethanol-lauroylsarcosine solution (Sigma, St Louis, Mo), centrifuging on a cesium chloride gradient, re-extracting with phenol-chloroform:chloroform-1-butanol, precipitating with ethanol, and concentrating by centrifugation.²⁶ After determination of the RNA yield and purity, polyadenosine-positive messenger (m)RNA was obtained by elution from an oligo(dT) cellulose column (Pharmacia, Piscataway, NJ).²⁷

A alternata cDNA was prepared from mRNA by using the Time Saver Cloning kit (Pharmacia) for the first and second strand synthesis. *EcoRI* linkers were ligated to the blunt ends of the cDNA and inserted in the unique *EcoRI* site of plasmid pZero (Invitrogen, San Diego, Calif). The cDNA was cloned into *Escherichia coli* Top10F's strain (Invitrogen). Expression of the lethal *ccB* gene was induced with isopropyl- β -D-thiogalactopyranoside in plating medium (Invitrogen), which resulted in the death of cells containing nonrecombinant vector.²⁸ Viable clones containing *A alternata* cDNA were therefore easily identified and selected for further analysis.

Screening of the cDNA library with human IgE antibodies

A crude cell lysate was prepared from the isopropyl-B-D-thiogalactopyranoside-induced bacterial cells. The cell lysate suspension was centrifuged at 20,000 rpm for 1 hour, and the supernate was collected. The total protein content of the lysate was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill).

IgE binding to recombinant *A alternata* proteins was detected by dot immunoblotting absorbed onto nitrocellulose membranes by using the dot-blot apparatus (BioRad, Hercules, Calif). Nonreactive sites on the membrane were blocked with 3% BSA in TRIS-buffered saline (TBS). The membranes were then incubated overnight with a 1:5 dilution of sera pooled from 12 *A alternata*-sensitive patients or control sera that had been preabsorbed with *E coli* extract. After washing with TBS containing 0.1% BSA and 0.05% Tween-20, a 1:1000 dilution of biotin-labeled goat anti-human IgE antibodies (KPL, Gathersburg, Md) was added. After incubation for 2 hours, washing as before, the membranes were incubated with a 1:5000 dilution of streptavidin-alkaline phosphatase conjugate (Pierce) for 1.5 hours. After washing, the substrates nitro blue tetrazolium (0.15 mg/mL) and 5-bromo-4 chloro-3 indolyl phosphate (0.3 mg/mL) in Sigma Fast (Sigma) were added. IgE binding was recognized by the appearance of a purple color on the membrane.

Studies involving human subjects had the approval of the University of Wisconsin-Madison Human Subjects Committee, and written informed consent was obtained from all participants.

DNA sequencing of the cDNA insert

Plasmid DNA was purified from a clone containing an *A alternata* cDNA insert, which demonstrated strong IgE binding, by using Magic Minipreps (Promega, Madison, Wis). Ten micrograms of DNA were digested with *KpnI*, *XhoI*, *XbaI*, and *SacI* endonucleases. After enzymatic digestion, the DNA was treated with exonuclease III (Erase-A-Base System, Promega) for the preparation of DNA nested deletions.²⁹ Sixteen time-point deletions were obtained at 15-second intervals at 25°C . After ligation with *EcoRI* linkers overnight at room temperature, *E coli* JM109-competent cells were transformed with DNA from each of the time-point deletions.²⁹

Plasmid DNA from each of the transformed cells was purified with Magic Mini-Prep (Promega). The annealing, labeling, and termination reactions were prepared by using the Sequenase Version 2.0 Kit (United States Biochemical, Cleveland, Ohio). The sequence of the *A alternata* cDNA clone was obtained by sequencing in both directions with the T3 and T7 universal primers.

High-level expression of rAlt a 2 in *Pichia pastoris*

After enzymatic digestion with *EcoRI* and *NotI*, cDNA inserts from positive clones were subcloned into *P pastoris* for high-level expression by using the vector plasmid pPIC9 (Invitrogen). The vector was inserted into *P pastoris* by means of electroporation. Twenty-five milliliters of buffered minimal glycerol-complex media (Invitrogen) were inoculated with the transformed *P pastoris* and incubated overnight at 30°C with vigorous shaking. One liter of buffered minimal glycerol complex was then inoculated with 10 mL of the overnight culture and incubated for 2 days under the same conditions. After 2 days, the cells were harvested by centrifugation and resuspended in 200 mL of media. The cells were then incubated at 30°C for 6 days with vigorous shaking. One milliliter of methanol was added to the culture every day as an inducer of protein synthesis.

After 6 days, the media was separated from the cells by centrifugation. The proteins in the supernate were precipitated by a 70% saturated NH_4SO_4 solution and collected by centrifugation. The resulting protein pellet was dialyzed overnight, lyophilized, assayed for protein content, and stored at -20°C .

Affinity purification of rAlt a 2 and native Alt a 2

To identify the presence of Alt a 2 in crude *A alternata* extracts, an affinity chromatography column was prepared with previously obtained murine mAbs raised against rAlt a 2. Three milliliters of AFFI-GEL (Bio-Rad) was coupled with 5 mg/mL mAb to rAlt a 2 according to the manufacturer specifications. Ten milligrams of lyophilized rAlt a 2 in 10 mL of PBS was absorbed into the column. The column was washed with 20 volumes of PBS and eluted with 0.1 glycine-HCl, pH 2.5.

Ten milliliters of crude *A alternata* extract (2 mg/mL) was absorbed onto a second affinity column. The column was washed and eluted as above to obtain purified native Alt a 2. Fractions from both columns were collected and analyzed in SDS-PAGE and Western blot.

Detection of IgE binding by individual sera from *A alternata*-sensitive subjects

One hundred microliters containing 1 mg/mL rAlt a 2 in TBS was absorbed onto nitrocellulose membranes by using the dot-blot apparatus (Biorad), and nonreactive sites on the membrane were blocked with 3% BSA as before. The membranes were incubated overnight with a 1:5 dilution of sera from 26 *A alternata*-sensitive

*Nucleotide sequence data of rAlt a 2 were submitted to the Genome Sequence Data Bank (GenBank) accession No. BankIt 55207 U62442. Approval of this new name was given by the International Union of Immunological Societies Allergen Nomenclature Sub-Committee on May 30, 1996.

subjects and 1 non-*A alternata*-sensitive control subject, which had been preabsorbed with nonrecombinant *P pastoris* media. After washing 3 times for 10 minutes with TBS containing 0.1% BSA and 0.05% Tween-20, a 1:5 dilution of iodine 125-labeled goat anti-human IgE antibodies was added, and the membranes were incubated overnight. After washing as before, the membranes were air-dried and then exposed to X-OMAT x-ray film (Kodak, Rochester, NY) for 48 hours.

Identification of rAlt a 2 DNA sequence in other *A alternata* strains

To determine whether DNA encoding for Alt a 2 is present in other strains of *A alternata*, genomic DNA from 3 *A alternata* strains (ATCC numbers 188699, A1002, and 11680) was isolated by the method of Lee and Taylor.³⁰ Two oligonucleotide primers encoding for the 5' and 3' ends of rAlt a 2 sequence were obtained from Operon Technologies Inc (Alameda, Calif). A 100 microgram PCR was conducted, which contained 20 mmol/L of each primer, 10 μ L of 10 $\times$ Taq buffer, 25 mmol/L MgCl₂, 25 mmol/L deoxyribonucleoside triphosphate, 2.5 units of Taq DNA polymerase (Perkin Elmer, Foster City, Calif), and 5 ng/reaction genomic DNA from the 3 *A alternata* strains. The reaction tubes were overlaid with mineral oil and amplified under the following conditions: 94°C, 54°C, and 72°C for 1 minute each for 30 cycles in a Coy Thermocycler (Ann Arbor, Mich). After amplification, the PCR products were resolved in a 1.0% agarose gel and stained with ethidium bromide.

RESULTS

Identification and isolation of a cDNA clone

The cDNA library contained 1.5×10^9 colony-forming units. The cDNA library was screened for IgE binding by using pooled sera (12 individuals) from *A alternata*-sensitive subjects. Twelve clones that showed IgE binding were identified. The length of the cDNA inserts from each clone as analyzed by electrophoresis in 1% agarose gel ranged from 0.4 to 2.2 kb. Clone 5, with the largest cDNA insert of 2.2 kb, was selected for further analysis because it had the most potential for yielding a complete gene sequence. cDNA from clone 5 was treated with exonuclease III for preparation of nested deletions and sequencing. The time-point deletions were analyzed and calculated to produce 125- to 150-bp deletions. By using the T3 and T7 primers, the full cDNA sequence of 2124 bp for the clone was obtained (Fig 1).

The cDNA sequence was analyzed with various computer programs available through Genetics Computer Group (Madison, Wis). Wordsearch (a sequence homology program) searched over 356,269 sequences in GenBank and the European Molecular Biology Laboratory databases. An open reading frame was located in the sequence between positions 1550 and 2120. An ATG starting codon is located 17 bp downstream from the Pribnow box, which is located at position 1534 to 1538. The termination codon, TGA, for this sequence is at position 2120.

The open reading frame has a total of 570 bp. The open reading frame sequence was also analyzed with the Translate and Peptidesort programs. A total of 190 amino acids were deduced from the cDNA sequence (Fig 1). The

polypeptide produced by the encoding cDNA sequence would be predicted to have a molecular weight of 22.3 kd as calculated by the Peptidesort computer program.

The cDNA sequence has homology with a common transposable region and mouse RNA-dependent EIF-2 α -kinase. The sequence does not encode for Alt a 1, Alt a 6, Alt 7, Alt a 10, enolase, or any other known allergen. The computer analysis also indicates that there are no mannose precursor tripeptides, such as the universal acceptors Asn-X-Thr or Asn-X-Ser, as potential N-glycosylation sites present in the amino acid sequence. The cDNA sequence has been entered into the GenBank database (accession No. BankIt 55207 U62442).

Expression and characterization of rAlt a 2

We elected to express rAlt a 2 in *P pastoris* because high yields of the recombinant protein can be easily obtained. The rAlt a 2 was purified by elution from an affinity column armed with murine mAbs to rAlt a 2. We obtained 180 mg of protein per liter of *P pastoris* culture media. Also, the native Alt a 2 was purified from a crude *A alternata* extract by using the same procedure. We obtained 0.7 mg of protein from each 10 mg of protein per milliliter of crude *A alternata* extract applied to the column. Both eluates were analyzed for molecular weight by SDS-PAGE with Coomassie blue staining. The purified rAlt a 2 has a molecular weight of 25 kd, and the native Alt a 2 has a molecular insight of 26.8 kd, as calculated by the relative mobility value of the molecular weight markers in the SDS gel (Fig 2). The rAlt a 2 and the native Alt a 2 were recognized by 4 mAbs to rAlt a 2 in a Western blot. Binding of mAb 10F2F8 to both rAlt a2 and native Alt a 2 was inhibited by incubating the mAb with crude *A alternata* extract (10 μ g protein/mL) before its application to the nitrocellulose strips. This suggests that mAb 10F2F8 recognized epitopes in the crude *A alternata* extract, as well as rAlt a 2 and native Alt a 2 (Fig 3).

The rAlt a 2 was analyzed for its ability to bind IgE by dot immunoblotting by using pooled sera from *A alternata*-sensitive individuals. The prevalence of IgE binding was also analyzed by using individual sera from 26 *A alternata*-sensitive subjects. Both a crude *A alternata* extract and rAlt a 2 gave strong IgE binding in the dot-immunoblot autoradiograph, whereas nonrecombinant *P pastoris* media and a BSA control did not show significant IgE binding (Fig 4). Sera from 16 of 26 (61%) *A alternata*-sensitive subjects showed significant IgE binding (Fig 5), which defines rAlt a 2 as a major allergen. Sera from a non-*A alternata*-sensitive individual did not show any significant IgE binding.

Analysis of genomic DNA from *A alternata* strains

DNA from 3 *A alternata* strains was amplified by using primers prepared from the 5' and the 3' end-terminal DNA sequence of rAlt a 2. The PCR products were resolved in 1.5% agarose gel stained with ethidium bromide and visualized under UV light. A 570-bp DNA

1532	ATTAAATCAAAAAGCAAAATGCATTGCTCCAATAATTTTTTAAGGATAA	1581
	M H S S N N F F K D N	
1582	TATATTTAGGTCGCTATCAAAGGAAGATCCTGATTACTCGAGAAATATAG	1631
	I F R S L S K E D P D Y S R N I E	
1632	AGGGTCAAGTCATAAGGTTGCATTGGGACTGGGCACAACCTCCTAATGCTG	1681
	G Q V I R L H W D W A Q L L M L	
1682	TCAGCTAAAAGAATGAAAGTAGCATTTAAGCTAGATATTGAGAAAGATCA	1731
	S A K R M K V A F K L D I E K D Q	
1732	ACGAGTTTGGGATAGATGCACAGCGGATGATCTTAAAGGGAGGAATGGTT	1781
	R V W D R C T A D D L K G R N G F	
1782	TTAAGCGATGTTTGCAATTTACCCCTTTACCGGCCAGGGATTACTATCA	1831
	K R C L Q F T L Y R P R D L L S	
1832	TTGTTGAATGAAGCTTTTTTTCCGCATTGAGAGAGAATAGAGAAACTAT	1881
	L L N E A F F S A F R E N R E T I	
1882	CATAAACACTGACCTAGAATATGCAGCCAAGTCAATTTCCATGGCCAGAC	1931
	I N T D L E Y A A K S I S M A R L	
1932	TTGAAGATCTCTGAAAGAGTATCAGAAGATCTTTCCTTCAATACAGGTT	1981
	E D L W K E Y Q K I F P S I Q V	
1982	ATAACTAGTGCATTTCTGTAGCATTGAACCTGAATTAACAGTTTATACGTG	2031
	I T S A F R S I E P E L T V Y T C	
2032	CTTAAAAAAATAGAAGCATCTTTTGAATTAATCGAAGAAAATGGAGATC	2081
	L K K I E A S F E L I E E N G D P	
2082	CTAAAATAACGTCTGAAATACAGTTGTTAAAGGCAAGTTGACC	2131
	K I T S E I Q L L K A S *	

-Pribnow box TAAAT position 1534-1538
 -Start codon ATG position 1550
 -Stop codon TGA position 2120

FIG 1. cDNA and deduced amino acid sequence for rAlt a 2. Nucleotide sequence (5' to 3') is for the Pribnow box, start codon, open reading frame, and stop codon. Deduced amino acid is in the open reading frame for the allergen. Stop codon TGA is denoted by an asterisk. Only the gene-encoding sequence is shown.

fragment was identified that was amplified from the genomic DNA from each of the 3 *A alternata* strains (Fig 6). These findings indicate that the sequence for rAlt a 2 is present in at least 3 other *A alternata* strains.

DISCUSSION

The diagnosis and treatment of *A alternata* sensitivity has been hampered by the lack of standardized allergenic extracts.⁹ Attempts to produce a standardized *A alternata* reference extract have been made.³¹ However, problems with stability of the extract arose that further emphasize the difficulty of working with these crude biologic preparations.

The development of DNA-based technology offers new approaches to the problem of standardization and

production of purified allergen in quantity. A number of important allergens have been molecularly cloned.³² Progress in the area of fungal allergy and with *A alternata* sensitivity has not been as rapid. Alt a 1 has been purified by biochemical techniques^{12,17-19} and its 20 N-terminal amino acid sequence has been reported.¹⁹ Subunits of Alt a 1 have been cloned.^{21,22} Recently, Achatz et al² molecularly cloned 3 *A alternata* allergens (Alt a 6, Alt a 7, and Alt a 10) and obtained their cDNA sequences. Computer searches of gene banks revealed that these allergens have functional activity. Alt a 6 is a P2 ribosomal protein that has also been found in *Cladosporium herbarum*. Alt a 7 showed sequence homology with YCP 4 yeast protein. Alt a 10 was identified as an aldehyde dehydrogenase. In addition, enolase is recognized as an important allergen from *A alternata*. It has been cloned

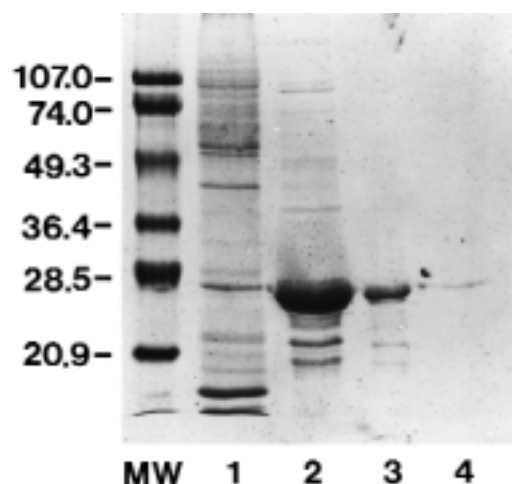


FIG 2. SDS-PAGE stained with Coomassie blue stain; molecular weight (MW) markers in kilodaltons. Lane 1, Crude *A alternata* allergen; lane 2, *P pastoris* media containing rAlt a 2; lane 3, affinity-purified rAlt a 2; lane 4, affinity-purified native Alt a 2.

from both *A alternata* and *C herbarium*, indicating it is a highly conserved fungal protein.²³

We have obtained the full cDNA sequence of a major *A alternata* allergen, rAlt a 2. On the basis of the reported N-terminal amino acid sequence, our gene would not encode for Alt a 1.¹⁹ Furthermore, we did not identify any homology with the cDNA sequences for Alt a 6, Alt a 7, Alt a 10,² or enolase.²³ DNA sequence homology was found to a common transportable region and to a mouse RNA-dependent EIF-2 α -kinase. The biologic relevance of these findings is not known.

Using SDS-PAGE, we have found our recombinant protein and the native Alt a 2 to have molecular weights of 25 and 26.8 kD, respectively. Using the Peptidesort computer program, we calculated that the polypeptide produced by the encoded cDNA sequence would have a molecular weight of 22.3 kD, which compares favorably with the SDS-PAGE results. At the present time, we do not know what posttranslational modifications to the polypeptide may have occurred to account for the mass increase. In comparison, purified Alt a 1 has been reported to have a molecular mass between 29 and 31 kD.¹⁷⁻¹⁹ The molecular masses of the 3 previously cloned *A alternata* allergens are 11 kD for Alt a 6, 22 kD for Alt a 7, 53 kD for Alt a 10,² and 46 to 48 kD for enolase.²³

We chose to express the recombinant protein from our cDNA insert in the yeast *P pastoris*. In contrast to expression of proteins in *E coli*, expression in the yeast system allows proteins to be glycosylated. Some *A alternata* allergens are known to be glycoproteins, and it has been postulated that IgE antibody recognition of *A alternata* allergens may in part be determined by carbohydrate moieties on the molecule.¹ However, computer analysis demonstrated that there are no mannose precursor tripeptides, such as the universal acceptors Asn-X-Thr or Asn-X-Ser, present in the deduced amino acid

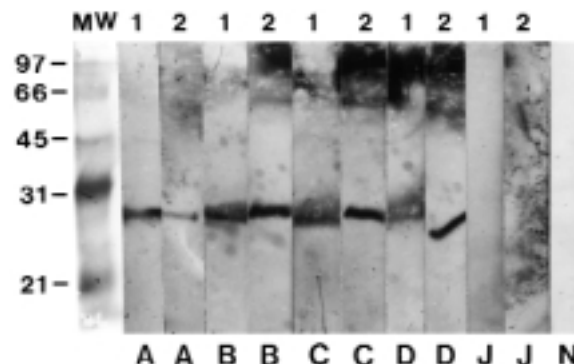


FIG 3. Western Blot of affinity-purified rAlt a 2 (strips 1) and native Alt a 2 (strips 2) probed with murine mAbs to rAlt a 2. A to D represent mAbs used (A, 6A11F5; B, 10F2F8; C, 8A2F12; D, 9E9F2); J represents inhibition of mAb 10F2F8 binding to rAlt a 2 and native Alt a 2 by crude *A alternata* extract (see text). MW, Molecular weight markers; N, negative control (no first antibody).

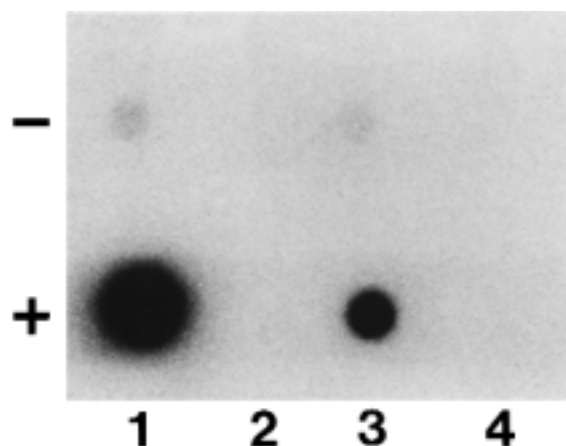


FIG 4. Dot-blot autoradiograph of 4 samples: 1, crude *A alternata* extract; 2, nonrecombinant control *P pastoris* media; 3, rAlt a 2; 4, BSA control. Blots were incubated with pooled sera from subjects sensitive to *A alternata* sera (+) or pooled control sera from non-allergic subjects (-). IgE binding was then detected by iodine 125-labeled rabbit anti-human IgE antibodies and autoradiography. All protein solutions were prepared at a concentration of 1 mg/mL. One hundred microliters was added to each well (dot).

sequence. This would suggest that there are no N-glycosylation sites in the protein. We further evaluated the recombinant protein for glycosylation by subjecting it to the enzyme N-glyconase (data not shown). SDS-PAGE analysis conducted on the recombinant protein before and after treatment with the enzyme did not show any significant shift in molecular weight, indicating that significant N-deglycosylation did not occur. Furthermore, IgE binding to the recombinant protein was not altered by N-glyconase treatment. We did not, however, assess for the possibility of O-glycosylation. Nonetheless, our results suggest that IgE binding is most likely directed against protein epitopes.

We also investigated the possibility of the rAlt a 2 sequence being present in other *A alternata* strains.

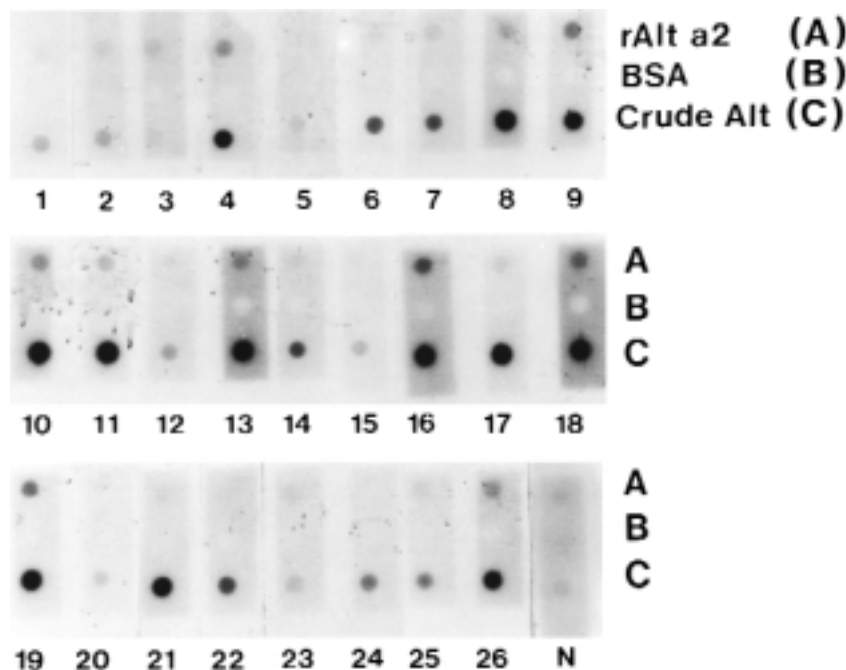


FIG 5. Dot-blot autoradiographs showing IgE binding of 26 individual human sera to *A alternata* proteins: A, rAlt a 2; B, BSA (irrelevant protein); C, crude *A alternata* extract; strip N, incubated with sera of non-*A alternata*-sensitive individual. All protein solutions were prepared at a concentration of 0.1 mg/mL. One hundred microliters was added to each well (dot).

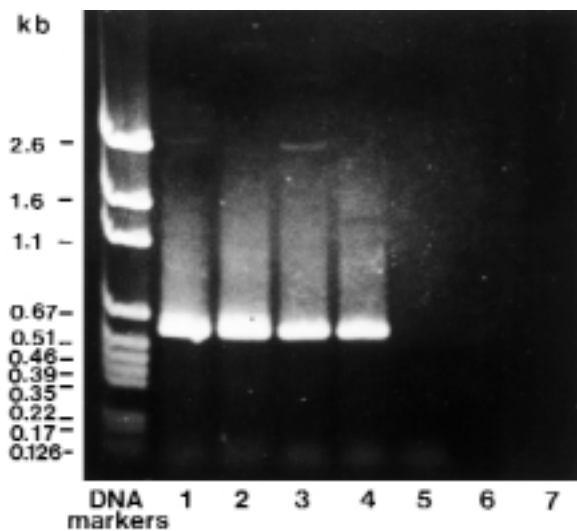


FIG 6. One percent agarose gel showing the amplified rAlt a 2 sequence in different *A alternata* strains. DNA markers, pGem DNA markers. *A alternata* ATCC strains: lane 1, 11680; lane 2, 188699; lane 3, A1002; lane 4, 46582; lane 5, no template; lane 6, no primers; lane 7, blank.

Using PCR techniques, we found that the rAlt a 2 sequence is also present in 3 other strains that we tested. Furthermore, a preliminary report from another group indicates the presence of the rAlt a 2 sequence in at least

3 other *A alternata* strains (ATCC 96154, 188700, ATCC 96152, and ATCC 96153).³³ These findings suggest that Alt a 2 is a conserved protein in *A alternata*.

The biologic significance of rAlt a 2 in *A alternata* sensitivity is yet to be fully determined. Clearly, IgE binding has been demonstrated in vitro to the recombinant protein. Moreover, we have found that 16 of 26 (61%) individuals with *A alternata* sensitivity had serum IgE antibodies to rAlt a 2, which indicates that it is a major allergen.

Fungal extracts are complex biologic materials. The production of allergens by *A alternata* varies depending on the strain of the organism and the culture media.¹¹ The low yield of native Alt a 2, which we obtained from crude *A alternata* extracts, demonstrates the difficulty in purifying *A alternata* and other fungal allergens. High-level expression of rAlt a 2 in *P pastoris* has enabled us to obtain the allergen in quantity and to explore its biologic significance. Although Alt a 2 comprises a fraction of the total protein in crude *A alternata* extracts, nonetheless it is a major allergen on the basis of its IgE-binding characteristics. Furthermore, the molecular cloning technology eliminates the variability of allergen expression by *A alternata* when grown on differing culture media and conditions.

The use of molecular biology techniques to produce *A alternata* allergens in quantity will be useful in the development of improved materials for diagnosis and therapeutic purposes. The reported high-level expression of a

major *A alternata* allergen, rAlt a 2, in *P pastoris* will facilitate this process. Additionally, a stable supply of a well-characterized major allergen will allow us to explore the role of this protein in the pathogenesis of *A alternata* sensitivity.

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